Coepibolin, the Activity of Human Serum That Enhances the Cell Spreading Properties of Epibolin, Associates With Albumin

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Earlier studies have shown that epibolin, a glycoprotein in human plasma, identical to preparations from other laboratories referred to as serum spreading factor and vitronectin, requires a second plasma activity in order to support maximal primary epidermal cell spreading. This plasma fraction, referred to as coepibolin, alone supports no cell spreading but potentiates the activity of epibolin by at least 5–10 times. Studies reported here indicate that coepibolin activity associates with one plasma fraction, it cannot be substituted by commercial preparations of ovalbumin, soybean trypsin inhibitor, or hemoglobin, but it can be substituted by commercial bovine or human serum albumin. The activity purified to electrophoretic homogeneity comigrates with, and shows antigenic properties of albumin. Under the assay conditions coepibolin affects the extent and morphology of cell spreading: in the presence of epibolin plus coepibolin cells assume a more polar orientation than in epibolin alone. For its maximal effect coepibolin must be present continuously in the early phases of cell spreading. *J Invest Dermatol 89:59–63, 1987*

It has long been recognized that plasma contains a property that supports the adhesion and spreading of cells in tissue culture. For mature, freshly isolated epidermal cells, one of those functions has been purified and identified as a 65 kD glycoprotein termed epibolin [1,2]. Recent studies have shown that epibolin appears to be identical to preparations put forth by other laboratories and referred to as serum spreading factor [3], vitronectin [4] and S-protein [5].

In the initial isolation studies, it was found that the specific spreading activity of crude epibolin did not increase after the DEAE chromatography step, even though inactive protein was removed [1]. Maximal specific activity was recovered, however, upon adding the inactive discard fraction from this step to the assay. Since the discard fraction alone did not support cell spreading, it was assumed that this fraction contained an activity potentiating that of epibolin. This activity was referred to as coepibolin. The present studies were conducted to further characterize the coepibolin activity of plasma and to probe its possible mechanism of action. These studies show that coepibolin activity is not a function of all proteins and that it purifies with albumin. Its action appears to modify the morphology of epibolin-induced epidermal cell spreading, and it appears to be needed by the cells continuously in order to produce an effect.

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Abbreviations:
BSA: bovine serum albumin
Con A: concanavalin A
DMEM: Dulbecco's modified Eagle's medium
HSA: human serum albumin
PAGE: polyacrylamide gel electrophoresis
P-buffer: 50 mM NaH_2PO_4/Na_2HPO_4, pH 6.0
STI: soybean trypsin inhibitor

MATERIALS AND METHODS

Cell Spreading Assay The direct spreading activity of all plasma fractions was assayed using trypsin-dissociated guinea pig epidermal cells as previously described [2]. To assure neutralization of residual trypsin the cells were rinsed once in a solution of Dulbecco's modified Eagle's medium (DMEM, GIBCO, Grand Island, New York) and soybean trypsin inhibitor (STI) (Sigma Chemical Co., St. Louis, Missouri) 1.0 mg/ml. Approximately 2×10^5 cells suspended in DMEM were added to tissue culture Petri dishes (Corning 34 × 10 mm) containing DMEM plus an appropriate dilution of the protein fraction to be assayed. After 4-h incubation (37°C, 95% air/5% CO_2, H_2O saturated) and Giemsa staining [6], the percent of spread cells (number of spread cells per 100 attached cells) was quantitated microscopically. A cell was considered to be spread if its cytoplasm showed a radial or polar pattern [7]. The coepibolin activity of a preparation was assayed by adding that fraction to culture medium containing epibolin at a limiting concentration that alone did not support significant epidermal cell spreading at 4 h. Maximal cell spreading was defined as the percent of cells spreading in the presence of 10% fetal calf serum. Under these conditions 40–60% of the adherent cells showed cytoplasmic spreading. One spreading unit was defined as the percent of spread cells (at 4 h) per milligram of protein assayed.

Protein Purification All chemicals were of reagent grade purchased from local suppliers. Water was singly distilled. All buffers contained 0.02% NaN_3, 0.1 mM phenylmethylsulfonyl fluoride (Sigma) (diluted from a 0.1 M stock solution in isopropyl alcohol), 20 mM ϵ-amino caproic acid, and 20 mM thioglycollate (Sigma). Bovine serum albumin (BSA, A4503), human serum albumin (HSA, A9511), transferrin, ovalbumin, hemoglobin, and STI were purchased (Sigma). Highly purified HSA was also purchased (Calbiochem 1266 58; Calbiochem, San Diego, California). Di-

alysis bags were prepared as described [1]. Using BSA as the standard, protein concentrations were determined by the method of Lowry et al [8].

Plasma was fractionated at 4°C as described [1] with minor changes. Briefly, to 100 ml of fresh human plasma collected from normal donors (Yale-New Haven Blood Bank, New Haven, Connecticut, within 1 week of bleeding) and cleared by centrifugation (5000 *g* for 20 min) (Beckman J 6-B centrifuge) was added 50 ml of saturated $(NH_4)_2SO_4$ with slow stirring for 1 h. The precipitate was collected by centrifugation (5000 *g*, 20 min, 4°C) and discarded. To the supernatant was added 150 ml of saturated $(NH_4)_2SO_4$ (to 2/3 saturation) and mixed for 2 h. This precipitate was collected (5000 *g*, 20 min, 4°C) and resuspended in 100 ml of 50 mM NaH_2PO_4/Na_2HPO_4, pH 6.0 (P-buffer) and dialyzed against the same (2 × 4 liters, 4°C, 24 h). The dialysand was poured over a DEAE Biogel A (100–200 mesh, BioRad, Richmond, California, 8 × 11 cm) column equilibrated with P-buffer. The wash effluent fractions of A_{280} absorbance more than 3.0 were pooled and labeled "coepibolin." Epibolin was further purified by washing the DEAE column retentate with P-buffer until the A_{280} was less than 0.05 and eluted by step gradient with P-buffer + 100 mM NaCl. The eluate fractions of A_{280} greater than 1.0 were pooled and added directly to a heparin Sepharose column (Pharmacia, Piscataway, New Jersey, 2 × 20 cm) previously equilibrated in P-buffer + 100 mM NaCl. The sample was washed with 100 ml P-buffer + 100 mM NaCl and then with 50 mM NaH_2PO_4/Na_2HPO_4 pH 7.2 + 100 mM NaCl until the effluent A_{280} was less than 0.05. The active material was eluted with 50 mM NaH_2PO_4/Na_2HPO_4 pH 7.2 + 500 mM NaCl and all fractions greater than 0.5 A_{280} absorption were pooled and frozen (−14°C). The epibolin activity of this preparation is completely blocked by a monoclonal antibody to human epibolin. This antibody was prepared using standard procedures [9], generated in defined medium [10], and used as the 50% saturated ammonium sulfate precipitate after dialysis against 50 mM Na_2H/NaH_2PO_4 pH 7.2 + 100 mM NaCl.

The fraction labeled "coepibolin" above (50 ml approximately 5 mg protein/ml) was further purified by titrating the solution with 1 N NaOH to pH 8.0 and applying it to a DEAE Biogel column 4 × 10 cm (4°C), which was equilibrated to 50 mM NaH_2/Na_2HPO_4 pH 8.0. The loaded column was washed until the A_{280} was less than 0.1, then washed with the same buffer + 100 mM NaCl until the A_{280} was less than 0.1. Coepibolin activity

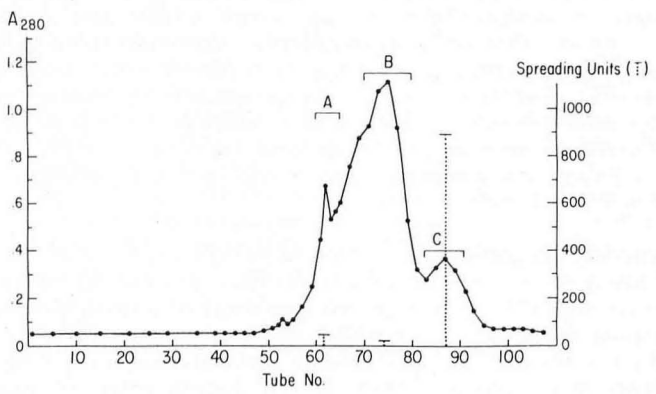

Figure 1. Coepibolin activity and gel filtration elution profile of "coepibolin" fraction. Two milliliters of the "coepibolin" preparation (see *Materials and Methods*) (11 mg/ml) was loaded onto a 2 × 80 cm column of Sephacryl S 200 (Pharmacia) at a flow rate of 10 ml/h at 24°C. The column was developed with a 20 mM Tris-HCl buffer pH 8.0 plus 1 M KCl and inhibitors (see *Materials and Methods*). The *left ordinate* reads protein concentration in A_{280} units. The *abscissa* gives protein elution tube number (2 ml fractions). The three protein peaks were pooled (as indicated by the *brackets*), labeled A, B, and C, and assayed for cell spreading (see *Materials and Methods*). In the assays epibolin concentration was 1–5 μg/ml and the tested coepibolin fractions 0.5–1.0 mg/ml.

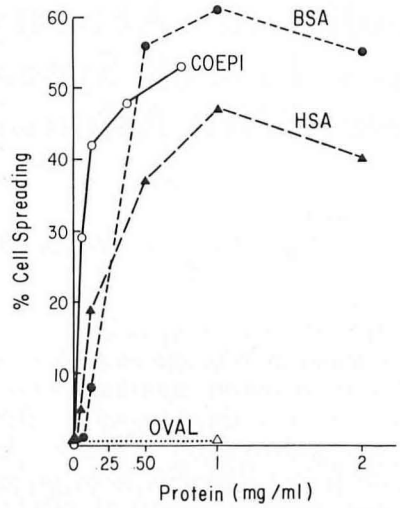

Figure 2. Coepibolin activity of various proteins over a concentration range (0–2 mg/ml). Bovine and human serum albumin (*BSA* and *HSA*, respectively) as well as coepibolin (*COEPI*) demonstrated epibolin-enhancing activity. In contrast, ovalbumin (*OVAL*) had no activity. Each point is the average of 2 experiments counting 200 cells in each. The SEM in these experiments was not more than 10%. In the assays epibolin concentrations were 1–5 μg/ml.

was eluted with 50 mM Na_2H/NaH_2PO_4 pH 6.0 + 100 mM NaCl. (This fraction was labeled A50P.) This eluate was immediately added to a concanavalin A (Con A) Sepharose column (Pharmacia 2 × 15 cm) and the wash collected (this fraction was labelled A50P-1). The retained protein eluted with 1 M alpha CH_3 mannoside (Sigma) in the same buffer had no coepibolin activity. One prominent and three faint bands were found after protein electrophoresis of the A50P-1 fraction using nondenatured polyacrylamide gel electrophoresis (PAGE) [11]. The bands were electroeluted by the procedure of Hunkapiller and colleagues [12] and assayed. That band running with an R_f value of 0.74 in the Davis PAGE system was the only one with coepibolin activity (A50P-1D).

Electrophoresis Electrophoresis was performed by the procedure of Davis [11] and Laemmli [13]. Transblots were performed and stained by the method of Towbin and associates [14] using nitrocellulose as the support, polyclonal rabbit antihuman albumin and polyclonal peroxidase labeled goat antirabbit antibody (Cooper Biochemical, Malvern, Pennsylvania), and 4 chloronapthol as the peroxidase substrate [15].

Table I. Coepibolin Activity of Various Proteins

Protein Additive	% Spread Cells
Epi (6)	5 + 3 (5)
Epi (40)	32 ± 1 (3)
Coepi (350	0 ± 0 (6)
Coepi (700)	0 ± 0 (4)
BSA (1000)	0 ± 0 (5)
HSA (1000)	0 ± 0 (5)
Epi (6) + Coepi (350)	47 ± 3 (4)
Epi (6) + BSA (1000)	42 ± 2 (4)
Epi (6) + HSA (1000)	47 ± 3 (3)
Epi (6) + Transferrin (1000)	0 ± 0 (3)
Epi (6) + Ovalbumin (1000)	0 ± 0 (3)
Epi (6) + Hemoglobin (1000)	2 ± 2 (3)
Epi (6) + STI (1000)	0 ± 0 (3)

The data are taken from 1 of 4 representative experiments. The epibolin (Epi) and coepibolin (Coepi) fractions were purified as described in *Materials and Methods*. The specified protein at concentrations in μg given in the parentheses was added to DMEM before cells were added. Spreading is expressed as the average number of spread cells per 100 attached cells ± SEM (number of cells × 10^{-2}) after 4 h incubation. BSA = bovine serum albumin, HSA = human serum albumin, STI = soybean trypsin inhibitor.

Table II. Stability of the Coepibolin Property of Human Serum Albumin (HSA)

HSA Treated	% Cell Spreading
Native	43 ± 2 (25)
Delipidated	37 ± 4 (12)
Heated 80°C 10 min	2 ± 1 (14)
Freeze-thaw 3×	36 ± 4 (13)
Dialyzed	31 ± 2 (13)
Trypsin treated	4 ± 1 (16)
Urea treated	35 ± 1 (5)
Guanidine HCl treated	38 ± 2 (5)
DTT treated	20 ± 4 (5)
NaOH exposed	0.3 ± 0.3 (3)
HCl exposed	21 ± 2 (5)

Cells were added to DMEM containing a limiting concentration of epibolin (1 μg/ml) and 1 mg/ml of the treated coepibolin preparation (HSA). The heated fractions were put into an 80°C water bath for 10 min. The freeze-thaw samples were cycled 3 times. The dialyzed samples were placed against a 100× volume of 10 mM Tris-HCl plus 0.85% NaCl pH 8.0 at 4°C overnight. Trypsin exposure was performed by adding 1 mg/ml trypsin (Sigma, crystalline T8253) to a 10 mg/ml HSA in P-buffer and incubating at 37°C. After 1 h trypsin was neutralized by addition of 1 mg/ml soybean trypsin inhibitor. Delipidated HSA was purchased (Sigma A3782). This preparation is delipidized by the method of Chen [16]. In separate experiments an HSA water solution (10 mg/ml) was exposed to urea (8 M, J. T. Baker Chemical Co., Phillipsburg, New Jersey), guanidine HCl (6 M, Heico, Inc., Delaware Gap, Pennsylvania), dithiothreitol (DTT, 10 mM, Sigma), NaOH (0.1 M), and HCl (0.1 M) for 60 min at room temperature. The NaOH and HCl experiments were neutralized with equivalent HCl and NaOH respectively, and all experiments were dialyzed against 100× volume of 10 mM Tris-HCl plus 0.85% NaCl pH 8.0 at 4°C overnight.

RESULTS

By Gel Filtration Chromatography Coepibolin Activity Associates With One Plasma Fraction The gel filtration elution profile of plasma coepibolin (prepared as described under *Materials and Methods*) is shown in Fig 1. The spreading activity of dissociated primary guinea pig epidermal cells was assayed in media containing limiting concentrations of epibolin. Virtually all the coepibolin activity associated with the last eluted protein peak (Fig 1, peak C). Coepibolin activity is thus not a nonspecific property of all plasma proteins.

Commercial Serum Albumin Contains Coepibolin Activity In Fig 2 the effect of cell spreading at varying concentrations of different proteins is plotted. It is seen that commercial BSA and HSA preparations are effective coepibolins. That not all proteins are coepibolins is again demonstrated by the inactivity of ovalbumin (Fig 2) and purified soluble proteins such as commercial transferrin, hemoglobin, and STI (Table I).

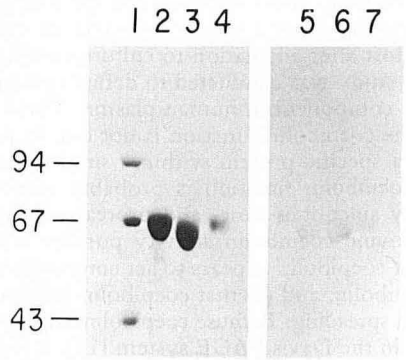

Figure 3. Electrophoretic and antigenic properties of human serum coepibolin fractions. Samples of purified coepibolin fractions isolated from human plasma (as described in *Materials and Methods*) were reduced (mercaptoethanol) and run on SDS PAGE [13]: protein standards with molecular weight (in kilodaltons) to the left (*lane 1*), fraction A50P-1 (*lane 2*), fraction A50P-1D (*lane 3*), and human serum albumin (Calbiochem) (*lane 4*) (*lanes 1–4*, 10 μg protein per well). Transblot electrophoresis and staining with rabbit polyclonal antihuman albumin is shown (fraction A50P-1, *lane 5;* A50P-1D, *lane 6;* and human serum albumin Calbiochem *lane 7*), (*lanes 5–7*, 25 μg protein per well).

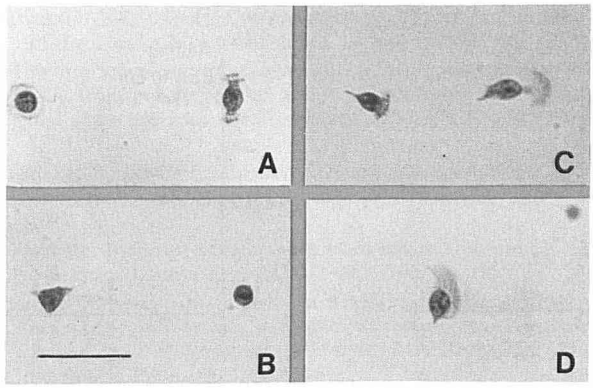

Figure 4. Photomicrograph of representative nonpolar and polar forms of dissociated epidermal guinea pig cells. Cells were cultured for 4 h in DMEM + epibolin (50 μg/ml) (*A, B*) and DMEM + epibolin (50 μg) + coepibolin (1 mg/ml) (*C, D*), stained with Giemsa [6] and photographed. *A* and *B*, A nonspread cell and spread cells with more than one plane of symmetry. *C* and *D*, Spread cells with only one plane of symmetry. *Bar* = 64 μm. Giemsa stain.

The Coepibolin Activity of HSA Is Destroyed by Heat, Proteolysis, and Dilute Alkali As shown in Table II, the coepibolin activity of HSA is stable to delipidation, freeze-thawing, dialysis, urea, and guanidine HCl; it is relatively stable to weak reduction or dilute acid, but it is destroyed by heat, trypsin proteolysis, and dilute alkali.

Coepibolin Activity of Human Plasma Purifies With Albumin As described in detail above (see *Materials and Methods*), the coepibolin fraction was further purified to electrophoretic homogeneity by DEAE Biogel chromatography at pH 8.0 (labeled fraction A50P), by Con A Sepharose chromatography (labeled fraction A50P-1) and by electroelution (labeled fraction A50P-1D). As shown in Fig 3, the purified electroeluted fraction stains as a single protein species, which has an electrophoretic mobility slightly faster than commercially prepared albumin but after transblotting shows albumin antigenicity. The difference in motility is ascribed to preparatory differences between the isolated (lanes 3 and 6) and the commercial (lanes 4 and 7) preparations, and the difference underscores the described microheterogeneity of albumin preparations [17]. Chromatography of the crude coepibolin preparation on Blue Sepharose (Pharmacia) also shows that the activity coelutes with albumin (experiments not shown).

The Presence of Coepibolin Appears to Influence the Shape of Spread Cells The spreading of cells in media containing epibolin alone was quantitatively less, but even under high epibolin concentrations, qualitatively different from that resulting in the presence of epibolin plus coepibolin. Figure 4 shows the representative cellular morphology of dissociated epidermal cells in the presence of epibolin alone (Fig 4*A,B*) or epibolin plus

Table III. Polar Spread Cells in the Presence or Absence of a Coepibolin Preparation

Protein Additive to Medium	Polar Cells/100 Spread Cells
Epi (24)	5 ± 1 (8)
Epi (1.2) + BSA (1000)	52 ± 2 (8)
Epi (1.2 + Coepi (1000)	46 ± 3 (4)

The conditions of assay were identical to Table I. A symmetrically spread cell showed more than one plane of symmetry; polar spread cells had only one plane of symmetry.

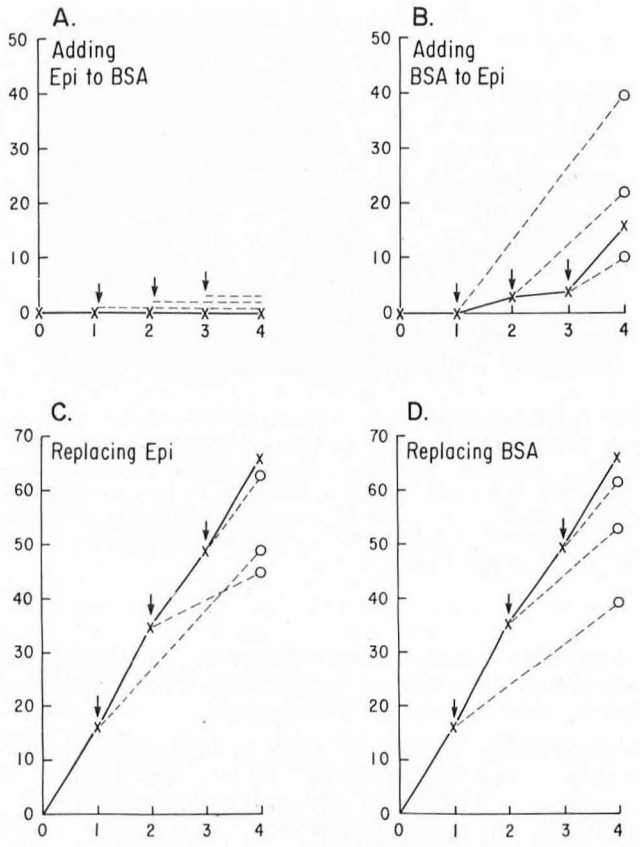

Figure 5. Spreading depends on the concomitant presence of epibolin and coepibolin. To test whether sequential exposure to epibolin or coepibolin is important, epibolin (1.25 μg/ml) or BSA (coepibolin) (1 mg/ml) was added or removed from a 4-h spreading assay. In all figures the *abscissa* represents time (h) and the *ordinate* percent of cells spreading. In the top figures either epibolin or albumin was added to the dishes previously containing one of these proteins. The *solid line* indicates cell spreading in the presence of either BSA alone (*A*) or epibolin (*B*). The *dashed lines* indicate cell spreading in media containing BSA and epibolin alone (right). The *arrows* indicate the time of the addition. In the bottom figures either epibolin (*C*) or albumin (*D*) was removed from dishes containing both of these proteins. At the *arrows* either epibolin (*C*) or BSA (*D*) was removed. The *solid line* indicates cell spreading in media containing both BSA and epibolin; and the *dashed lines* indicate spreading in media lacking one of the proteins. Each point represents the average of 2 experiments counting 8 × 100 cells (SE less than 10%).

coepibolin (Fig 4*C,D*) at 4 h. In epibolin alone the cytoplasm of the adherent cells appeared to spread in a more symmetric, often radial distribution. In contrast, cells spreading in the presence of epibolin plus coepibolin showed an asymmetric, often polar distribution. The morphology of spreading under these two conditions was quantitated by defining a radial spread cell as one with more than one plane of symmetry (as seen en face) and a polar spread cell as a cell with only one plane of symmetry. As given in Table III, the ratio of polar spread cells to total number of spread cells in the presence of coepibolin is 10 times more than is found in its absence. It is notable that an adherent cell passes in time from a radial to a polar form in the process of spreading in vitro [7]. Thus, this morphologic difference in the presence or absence of coepibolin may be due to a difference in the mechanism of spreading or to the rate of progression through the phases of spreading. At a 24-h time period, however, we have not found that the fraction of polar spread forms increases in the absence of coepibolin (data not shown); therefore, it appears likely that the mechanisms of cell spreading under these two conditions differ.

For Maximal Spreading Epibolin and Coepibolin Must Be Present Continuously To determine whether continuous exposure of cells to epibolin with and without a coepibolin was necessary for maximal spreading, several experiments were done. In one set of experiments, the cells were added to a tissue culture dish containing defined medium plus BSA (the coepibolin), and at hourly intervals epibolin was added (Fig 5*A*). In the second, the inverse experiment, the defined medium contained epibolin, and BSA was added at hourly intervals (Fig 5*B*). Adding epibolin to a BSA-containing dish after 1, 2, or 3 h resulted in no spreading. This finding is likely due to the blockage of epibolin binding sites on the dish by BSA as suggested previously [2]. Starting with epibolin and adding BSA after 1 and 2 h showed a significant increase in spread cells by the end of the 4-h incubation period. Adding BSA to an epibolin dish after 3 h led to insignificant cell spreading. In the experiment of Fig 5*C* and *D*, cells were added to defined media containing both epibolin and coepibolin. At hourly intervals media were completely replaced with either epibolin-free (Fig 5*C*) or coepibolin-free (Fig 5*D*) medium, and the spread cells were counted after 4 h. As shown, if either protein was removed at 1 or 2 h, less than maximal spreading was obtained. If the proteins were removed at 3 h the cells spread as well as in the undisturbed complete medium. These studies suggest that both components of this system must be present together for the first 3 h in order to achieve maximal spreading by 4 h and that one component does not act before, or independent of, the other. However, after 3 h in a medium containing both epibolin and coepibolin cells subsequently spread despite the removal of either component. Although the latter could be explained by time-dependent protein adhesion to plastic, it is also possible that the components were inadequately removed. The latter possibility is not favored because removal of either component at 1 h or 2 h (Fig 5*C,D*) led to less than maximal spreading.

DISCUSSION

From the comparative methods of purification, immunologic studies [3], and N-terminal amino acid sequence data (K. S. Stenn, manuscript in preparation), it appears that epibolin is identical to other preparations referred to as serum spreading factor [3], vitronectin [4,18], and S-protein [5,19]. Serum spreading protein has been found to support the spreading of numerous differentiated and transformed animal cell types in serum-free culture medium [20]. Studies of epibolin have been confined to the spreading of primary epidermal cells [1,2]. In apparent contrast to other cell types, primary (i.e., cells not previously passaged in culture) epidermal cells require a second plasma component (termed coepibolin) in order to effect maximal cell spreading. Coepibolin dependence may be either a unique property of epidermal cells or a property lost after adaptation to culture conditions.

The present study was conducted to define some properties of the coepibolin component in human plasma. These studies suggest (1) that the coepibolin function is not due to protein per se but is due to a specific protein within a single plasma fraction; (2) that the coepibolin function is probably associated with a protein moiety since it is heat- and protease-sensitive; (3) that human and bovine coepibolin activity purifies with serum albumin; (4) that coepibolin appears to act conjointly and concomitantly with epibolin; and (5) that coepibolin influences the morphology of cell spreading. Because coepibolin activity electroelutes with albumin in the Davis PAGE system [11], it is reasonable to conclude from the purification studies that coepibolin activity is due to (1) the protein structure of albumin or (2) a tightly adherent ligand of albumin. In either case coepibolin activity is relatively stable to denaturants but sensitive to protease and dilute alkali. These studies do identify the active molecular species.

Earlier studies showed that tumor-promoting phorbol esters completely substitute for coepibolin [21]. Phorbol esters affect multiple cellular processes, but specifically they have been shown to produce profound changes in the cytoskeleton [22–26]. In fact, phorbol esters have been shown to induce a more spindled or

polar morphology in chick embryo fibroblasts reminiscent of the coepibolin effect [24] observed here.

That coepibolin may act like the phorbol esters through protein kinase C [27,28] is an attractive hypothesis currently being tested.

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